OCCURRENCE OF MELANIN IN BRIGHT-SPORED MYXOMYCETES

Indira KALYANASUNDARAM, Lata MENON and P. LOGANATHAN

Centre for Advanced Study in Botany, University of Madras,
Guindy Campus, Madras 600 025, India

ABSTRACT - In a study of spore wall pigments of eleven species of the Orders Liceales and Trichiales - taxa which are traditionally separated from the dark-spored Orders Physarales and Stemonitales by their variously coloured spores - it was found that even in these bright-spored Orders, melanin was present in the spore walls of all the species. In addition, there were several organic-solvent-extractible pigments, usually in low amounts.

RÉSUMÉ - Ce travail a eu pour but l'étude des pigments pariétaux de 4 espèces appartenant aux ordres des Liceales et des Trichiales, taxons habituellement isolés des Physarales et Stemonitales, ordres à spores pigmentées, en raison de leurs coloration variable. Il a été démontré que les parois des spores de ces 4 espèces, à pigmentation claire, contenait de la mélanine. En plus, la présence en faible quantité de pigments extractibles par les solvants organiques a pu être démontrée.

KEY-WORDS - Myxomycetes, spore pigments, melanin, Liceales, Trichiales.

INTRODUCTION

The occurrence of melanin as the only pigment in the spore walls of Myxomycetes belonging to the dark-spored orders Physarales and Stemonitales, has been reported (Loganathan *et al.*, 1989). We studied the spore wall pigments of some species belonging to the Liceales and Trichiales - traditionally the bright-spored group - on which relatively new studies have been made (Liaane-Jenson, 1965; Blackwell & Busard, 1978; Czeczuga, 1980; Steglich *et al.*, 1980; Kopanski *et al.*, 1982, 1987).

MATERIALS AND METHODS

Materials

For extraction of pigments, spores are required in large quantities. The Liceales and Trichiales generally favour temperate climates. Specimens were collected by the first author in the foothills of the Himalayan mountains in the state of Himachal Pradesh (H.P.), at 31°15' to 31° 45' N latitude, 77° 25' to 77° 60' longitude, at elevations of 2300 to 3000 m, in September 1989. They were identified after Lister (1911), Martin & Axelopoulos (1969) and Emoto (1977). Twelve species were selected for study. Spore colours were determined with reference to Rayner (1970). Details of

the material are listed in Table I. One species of the Stemonitales was included for comparison.

Table I - Material used

Order and specific name	Herbarium N°	Source
LICEALES		
**Lycogala epidendrum* (L.) Fries	MUBL/IK/FC/185	Fresh collection from H.P. (a)
Reticularia lycoperdon Bull.	HPUB/12750	From the collection of Dr TNL (b)
Cribraria atrofusca Martin & Lovejoy	MUBL/IK/FC/194	Fresh collection from H.P.
TRICHIALES		
Arcyria ferruginea Fuckel	HPUB/12604	From the collection of Dr TNL
A. nigella Emoto	MUBL/IK/FC/182	Fresh collection from H.P.
**A. occidentalis* (Macbr.) G. Lister	MUBL/IK/FC/176	"
A. stipata (Schw.) A. Lister	MUBL/IK/FC/178	"
**Trichia decipiens* (Pers) Macbr.	MUBL/IK/FC/173	"
T. favoginea Schum.	MUBL/IK/FC/170	"
T. varia (Pers.) Pers.	MUBL/IK/FC/172	"
**Hemitrichia serpula* (Scop.) Rost.	MUBL 2882	Earlier collection from Coorg, Karnataka
STEMONITALES		
Stemonitis fusca Roth	MUBL/IK/FC/196	Fresh collection from H.P.

* Non-melanin pigments also studied
(a) Himachal Pradesh
(b) Prof. Dr T.N. Lakhanpal, Biosciences Department, H.P. University, Shimla.

Extraction of pigments

Whole spores were used for all extractions. These were separated by manually shaking dry sporangia in small closed boxes until the sporangial structures separated from spores, rolled up into a ball and could be removed *en masse*. The spores were collected in small test tubes, sealed and stored at 4°C until needed. Non-melanin pigments were extracted only from four species (marked with an asterisk in Table I), the amount of spores collected from the others being insufficient for two types of extraction.

Melanin was extracted by standard methods (Thomas, 1955) from pre-weighed spores with 1 M KOH, and purified as detailed earlier (Loganathan *et al.*, 1989). After drying, the weight of this purified pigment was determined. Non-melanin pigments were extracted by boiling about 10 mg of spores in 5 ml of ethanol in a closed tube for 1 h. After cooling, the material was centrifuged at 2000 rpm and the supernatant collected. The process was repeated until no more pigment could be extracted. The extracts were pooled and concentrated over a water bath. As very little pigment could be extracted by this method, the method described by Steglich *et al.*, (1980) was tried with *Trichia decipiens*, of which there was a plentiful supply. Following ethyl acetate,

several other solvents such as methanol, acetone, diethyl ether and dimethyl sulfoxide were tried in succession, but only part of the pigment could be extracted and the spores still remained coloured.

Analysis

Melanin was subjected to the various physical and chemical tests as detailed in Loganathan *et al.* (1989).

Non-melanin pigments were separated by thin-layer chromatography on silica gel-G (Merck). The plates were developed with Toluene:Formic acid-ethyl acetate:Formic acid (10:10:3) (Kopanski *et al.*, 1982). The spots thus separated, however, were too faint in most cases for further elution and testing. The chromatographs of *T. decipiens* were developed in methanol, after initial testing with the above solvent system and a few others. A single spot which developed from each extract, was eluted and read in a Beckmann DU-40 spectrophotometer at the range of 200 to 500 nm.

RESULTS

Melanin

A brown pigment was extracted with 1 M KOH from all the 12 species, and a range of tests (Loganathan *et al.*, 1989) showed the pigment in all the species to be melanin.

The ultra-violet spectra of the melanins from the 12 species showed a fairly uniform pattern (Fig. 1). The Liceales showed the highest absorption at 220 to 223 nm, as most clearly exemplified in *Cribraria atrofusca*. In the Trichiales the peaks were also seen at 221-223 nm, and were sharply defined in all species. A hump at 280 to 310 nm, and were sharply defined in all species. A hump at 280 to 310 nm was seen in most of the species in both the orders, but it was not very prominent except in the Trichias and in *Hemitrichia serpula*. In *Stemonitis fusca*, the absorption peak was seen at 222 nm.

The infra-red spectra of the melanins of the 12 species showed variations (Fig. 2). The prominent characteristics were:

a. Sharp absorption peaks at 3.3 to 3.4 μm (2860 & 2930 cm^{-1})
b. A broad band at 4.3 to 5.0 μm (2000-2400 cm^{-1})
c. An absorption peak at 5.7 to 6.0 μm (1715 cm^{-1}).

In addition to these, absorption at 4.1-4.3 μm (2300-2500 cm^{-1}) was uniformly seen in all the 12 species, but this was never prominent. Based on the presence or absence/ prominent or diffuse nature of these features, the 12 species could be broadly separated into four groups, but this grouping had no bearing on their taxonomic status (Table II).

The percentage of melanin in relation to the fresh weight of the spores was determined only in two species of the Liceales, amounting to 7-10%, and four species of the Trichiales amounting to 4.5% in *Trichia* and 3% in *Arcyria* (Table III). The dry weight of the spores was determined only in *T. decipiens* where a separate extracion gave 6.8% melanin.

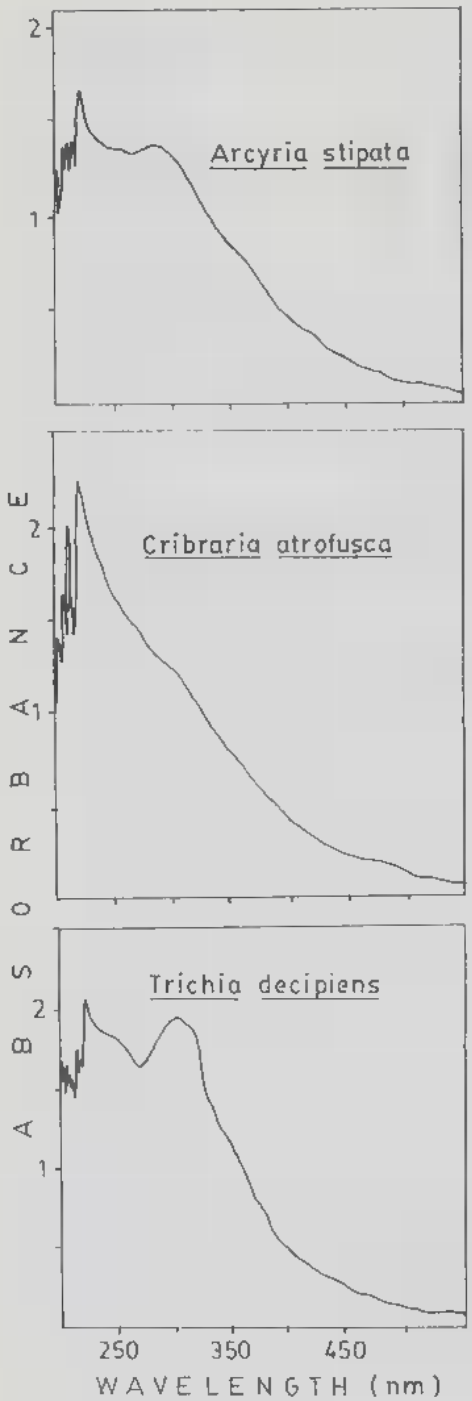

Fig. 1 - Ultraviolet spectra of melanin from three representative species of the Trichiales and Liceales, showing characteristic absorption patterns.

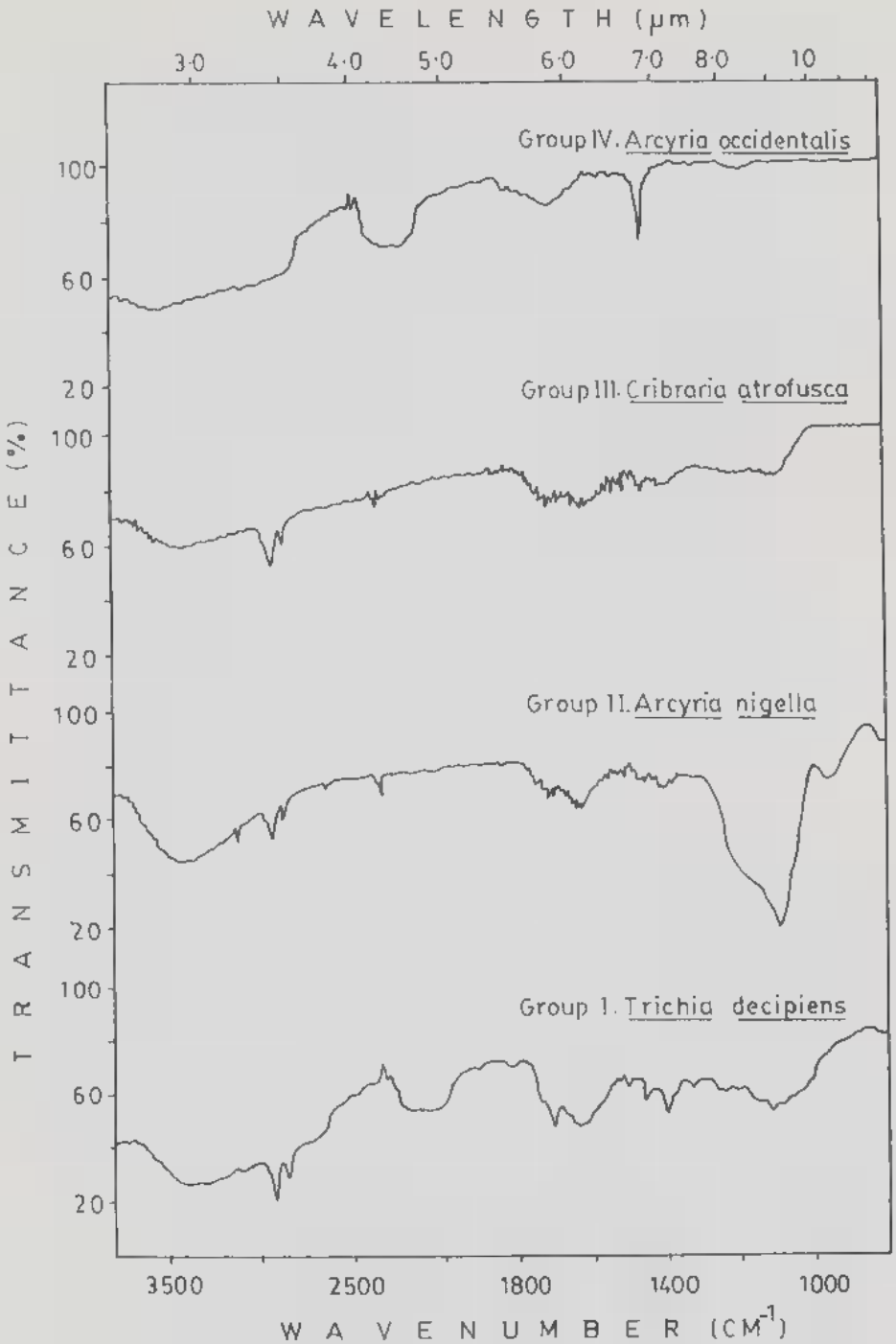

Fig. 2 - Infra-red spectra of melanin from the Trichiales and Liceales, representing the four groups described in the text.

Table II - Infra red patterns of melanins of Liceales, Trichiales and *Stemonitis*.

Grouping	species	Features			
		a Peaks at 3.3-3.4 µm	b Trough at 4.3-5.0 µm	c Peaks at 5.7-6.0 µm	Remarks
I	*Lycogala epidendrum*	+	+	+	
	Trichia decipiens	+	+	+	
II	*Arcyria stipata*	+	-	+	
	A. nigella	+	-	+	Additional peak at 9µm
	Trichia favoginea	+	-	+	
	T. varia	+	-	+	
	Stemonitis fusca	+	-	+	
III	*Cribraria atrofusca*	+	-	-	
	Reticularia lobata	+	-	-	
	Arcyria ferruginea	+	-	-	
IV	*Arcyria occidentalis*	-	+	Diffuse	Additional sharp peak at 7 µm
	Hemitrichia serpula	-	+	Diffuse	

Table III - Summary of spore wall pigments in the twelve species studied.

Order and Species	Spore colour* (Nearest shade: Rayner)		Melanin	Percentage (W/W) (Fresh wt)	Number of other pigments in ethanol extract
	Term	Notation			
LICEALES					
Lycogala epidendrum	Buff	19''f	+	10.0	6
Reticularia lobata	Dark brick	7''k	+	-	-
Cribaria atrofusca	Umber	13 m	+	7.0	-
TRICHIALES			+		
Arcyria ferruginea	Bay	5 k	+	-	-
A. nigella	Sepia	13''k	+	3.0	-
A. occidentalis	Umber	13 m	+	-	4
A. stipata	Fawn	11''	+	3.0	-
Trichia decipiens	Umber	13 m	+	4.5	3
T. favoginea	Amber	19'	+	4.5	-
T. varia	Ochreous	13'b	+	-	-
Hemitrichia serpula	Umber	13 m	+	-	5
STEMONITALES					
Stemonitis herbatica	Chesnut	5'm	+	-	None

+ : present; - : not done

* In a few cases, spores were somewhat faded at the time of colour determination.

Non-melanin pigments

The solvents for pigment extraction and for developing the chromatographs were selected after preliminary trials with several solvent combinations. Ethanolic extracts were analysed from *Lycogala epidendrum, Trichia decipiens, Arcyria occidentalis* and *Hemitrichia serpula*. For the other species, the spores were used up for melanin extraction. Boiling with ethanol did not give complete extraction, as the spores still remained coloured. The pigments of *T. decipiens* were extracted with a series of solvents but the spores still remained coloured. Chromatographic separation of the extracts revealed six fractions in *Lycogala*, three in *Trichia*, four in *Arcyria* and five in *Hemitrichia*, with Rf values ranging from 0.2 to 0.9. The UV absorption of the pigments extracted from *Trichia* in different solvents is presented in Table IV. When the dried pigments were pooled together, they formed 7.5% of the dry weight of spores.

Table IV - UV absorbance of pigments extracted from *Trichia decipiens*.

Solvent	Absorbance at (wave length in nm)				
	230	250	270	290	300
Ethyl acetate	-	+	-	-	-
Dimethyl sulfoxide	-	-	+	-	-
Diethyl ether	+	-	-	+	-
Ethanol	-	+	-	-	+
Water	+	-	-	-	-

DISCUSSION

Melanin

Although melanin has previously been reported in a Liceaceous species (Loganathan *et al.*, 1989), its occurrence in all the other species including the bright-spored Arcyrias and Trichias, was a surprise. Melanin forms 3 to 10 per cent of the fresh weight, or up to 7 per cent of the dry weight of whole spores. In the dark-spored orders where melanin is the only pigment, it constitutes 7 to 15 per cent of the dry weight of separated spore walls (McCormick *et al.*, 1970; Chapman *et al.*, 1983; Paramasivan, 1990). From our results in *Trichia decipiens*, there is an indication that the melanin and non melanin pigments may occur in equal amounts.

Apparently, in the bright-spored species, melanin is masked by other pigments. Sporangia of Trichias, still in the process of development, are of a shiny black colour and if crushed, would exude the still semi-liquid contents as a purplish-black fluid. The characteristic brownish yellow colour of the mature sporangia develops much later. The non-melanin pigments could be extracted from spores before, but not after the extraction of melanin, apparently being destroyed by the drastic procedures of melanin extraction. It seems reasonable to presume that the bright pigments appear after the completion of melanin synthesis, and that they occur at the surface.

The UV spectra of melanin from both the Liceales and Trichiales showed a fair degree of uniformity, with peaks at 221-223 nm. In this respect they were similar to the melanins of the Stemonitales (Loganathan *et al.*, 1989). Of the hump at 280-310 nm, seen in several species, there had been only a faint suggestion in the Physarales and Stemonitales.

The i.r. spectra showed similarities to and differences from those of the Physarales and the Stemonitales. The absorption around 3 μm (2860 & 2930 cm^{-1}), which was seen in all but two species, had been seen in both the dark-spored orders, although it was not equally prominent in all the species. The broad absorption at 4-5 μm, seen in *Lycogala epidendrum*, *Trichia decipiens*, *Arcyria occidentalis* and *Hemitrichia serpula*, and as low bands in all the species, had been seen as well-defined peaks in all the Physarales and Stemonitales (Loganathan *et al.*, 1989). The peak at 1715 cm^{-1}, clearly seen in *Lycogala epidendrum* and *Trichia decipiens* and in a diffuse way in some Arcyrias and Trichias, had been seen in all the members of Physarales and the Stemonitales.

The absorption at 9 μm (1000-1300 cm^{-1}), seen in *Arcyria nigella*, is similar to that described by Rast *et al.* (1981) as being characteristic of the GDHB melanin of *Agaricus bisporus*, and we have reported it earlier in *Reticularia lycoperdon* (Loganathan *et al.*, 1989).

Non melanin pigments

The separation into 3 to 6 fractions in our study is thus in accordance with earlier findings. Blackwell & Busard (1978) reported 3 to 6 fractions in the ethanolic extracts of some Trichiaceous species, with considerable intra- and interspecific variation. Steglich and his associates obtained several fractions from *Arcyria denudata* (Steglich *et al.*, 1980), *Trichia floriformis* and *Metatrichia vesparium* (Kopanski *et al.*, 1982, 1987). Czeczuga (1980) reported several carotenoid pigments from each of eight species of Myxomycetes, which represented all the four major orders. In the earlier studies, however, whole sporangia were used for pigment extraction and not the spores alone. The chemical nature of these pigments has been definitely established only in a few cases, through the studies of Steglich and his associates (Steglich *et al.*, 1980; Kopanski *et al.*, 1982, 1987).

Considering that the yellow and brown pigments of the Liceales and Trichiales become non-extractible after melanin extraction, and are not completely extractible even before melanin extraction, it is possible that they occur closely bound to the melanin molecule. Verification of such a hypothesis, however, has to await further study.

ACKNOWLEDGEMENTS

We are very thankful to Prof. T.N. Lakhanpal of the H.P. University, Shimla and his students Meera Thakur, Lal Singh Chauhan and Anand Sagar and to Mr and Mrs K.S. Thakur of the H.P. Forest Service, for the help extended to the first author in the collection of specimens; to our colleagues P. Jayaraman and N. Mubarak Ali for helping us in different ways; and to the University Grants Commission, New Delhi, for the award of a grant (F. 3-44/86) to the first author. The third

author thanks the Council of Scientific and Industrial Research, New Delhi for the award of a Senior Research Fellowship.

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